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Characterization of two *Achromobacter xylosoxidans* isolates from patients with pertussis-like symptoms

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ABSTRACT

Objective: To characterize two *Achromobacter xylosoxidans* recovered from 2 patients diagnosed with pertussis during a *Bordetella pertussis* surveillance program.
Methods: Nasopharyngeal swabs from 2 children under 1 year of age with clinical suspicion of pertussis were analyzed by culture and PCR.
Results: Two *Achromobacter xylosoxidans* A8, closely related to *Bordetella* spp. were recovered from 2 patients diagnosed of pertussis, both carrying the *ptxA* gene and *IS418* the pertussis toxin encoding gene. Subsequently, antibiotic susceptibility was evaluated by disk-diffusion method and by PCR.
Conclusions: Although more detailed studies are needed, the present data highlight the possibility that *Achromobacter xylosoxidans*, closely related *Bordetella pertusssis* microorganisms and not covered under the vaccine umbrella, might also result in cases of whooping cough. Thereby further surveillance is necessary to determine the extension and relevance of their pathogenic role in order to discriminate their real public health implication.

1. Introduction

Respiratory tract infections are the leading cause of mortality among children under five years of age worldwide, causing around 1.5 millions of deaths yearly, especially in low and middle income countries (LMC) [1]. Among these infections, whooping cough (pertussis) is particularly severe, with the WHO reporting more than 16 million cases of pertussis and around 195 000 deaths worldwide in 2008, 95% of which were in LMC [1]. Pertussis is caused by *Bordetella pertussis* (*B. pertussis*). It is potentially preventable by vaccination and is characterized by a series of early symptoms which may be easily misdiagnosed as a common cold, followed by a series of paroxysmal symptoms at 1 or 2 weeks of evolution, which may include coughing, whooping sounds, vomiting among others that might result in death [2].

The introduction of the pertussis vaccination resulted in a decrease in disease burden, but since the 80–90's of the last century, the number of cases has risen again [3,4]. This re-emergence of pertussis may be related to different causes, including less effectivity of the current acellular pertussis vaccine with respect to the whole cell vaccine [4,5], the selection of variants of *B. pertussis* not covered by the vaccine [5,6], as well as the underestimated role of other *Bordetella* species, as such as *Bordetella holmesii* [7]. Following these trends, in the last years an increasing number of cases, mainly in rural areas, has been described in Peru [8].

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The members of the *Achromobacter* genus are ubiquitous, being detected in different sources, including water environments or different human microbiotas [9,10]. *Achromobacter* is a genus closely related to *Bordetella* and it has been proposed to have a potential virulence from an evolutionary point of view [11]. In this line *Achromobacter xylosoxidans* (*A. xylosoxidans*) has been described as a cause of different infections, mainly bacteremia in immunosuppressed patients, but has also been related to respiratory diseases, including infections in patients with cystic fibrosis, bronchiectasis and pleural emphysema, among others [10,12–15]. However, to our knowledge it has never been reported as a causal agent of pertussis-like illness.

The aim of this study was to characterize 2 *A. xylosoxidans* isolated from pediatric patients diagnosed with whooping cough.

2. Materials and methods

2.1. Samples and clinical data

Nasopharyngeal swabs from 2 children under 1 year of age with clinical suspicion of pertussis were collected during a surveillance of pertussis and sent to the Instituto de Investigación Nutricional/Universidad Peruana de Ciencias Aplicadas. Relevant clinical data were recorded in a questionnaire. The study was approved by the Ethical Committee of the Hospital Nacional de Salud del Niño, Lima (Peru). The samples were collected after the parents had provided signed informed consent.

Clinical and epidemiological features were registered for each patient, including: age, clinical symptoms (paroxysm of coughing, cyanosis, respiratory distress, difficulty feeding, fever, flush, diarrhea, vomiting, wheezing, runny nose and expectoration), days from the onset of symptoms and time elapsed until the sample was collected and sent to the laboratory.

2.2. Clinical diagnosis

A direct nucleic acid extraction was performed from the nasopharyngeal samples using the High Pure Template Preparation Kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions, and pertussis status was confirmed by PCR. Thus, both 191 bp of the *ptxA* gene and 145 bp of the *IS418* were amplified using primers described elsewhere [16,17]. To confirm the correctness of the amplifications the PCR products were recovered, purified (SpinPrepTM Gel DNA Kit, San Diego, USA) and sequenced (Macrogen, Seoul, Korea). Additionally, the presence of adenovirus, influenza A, B and C, syncytial respiratory virus A and B, parainfluenza 1, 2, 3, 4a, enterovirus, rhinovirus and coronavirus was verified as described elsewhere [18].

2.3. Bacterial culture and identification

The nasopharyngeal samples were cultured in Bordet-Gengou agar, incubated at 35 °C in a moist atmosphere and maintained for 5–7 days under aerobic conditions. Colonies were identified based on characteristic morphology, Gram stain appearance and catalase, oxidase testing. Positive cultures were confirmed by PCR of the *ptxA* gene as mentioned previously. Additionally, a DNA fragment of 663 bp, including the first 517 bp of the *parC* gene, was also amplified using the primers (5′-GTGATCATTCCCAAGCGCC-3′ and 5′-TGAGCAGCATCACGGGCA-3′) and the conditions previously described by Ohtsuka *et al* [19]. These primers are able to amplify the *parC* gene from both *Bordetella* spp and *A. xylosoxidans*, which may be easily differentiated by the presence of several additional nucleotides in the *N*-terminal region of the *parC* gene of *A. xylosoxidans* sequence.

2.4. Antibiotic susceptibility

The susceptibility levels to amoxicillin, azithromycin, chloramphenicol, cotrimoxazole, erythromycin, clarithromycin, cefotaxime, ampicillin, moxifloxacin and tetracycline were performed by disk diffusion.

2.5. Macrolide target study

The *23S rRNA* gene was amplified from bacterial DNA extractions using the primers and conditions described previously [20]. Additionally, the *rplV* and *rplD* genes from both nasopharyngeal swabs and bacterial origins were amplified using *Bordetella*-specific primers: *rplV*-F: 5′-CGGACA AGAAGTCGAAGAGG-3′, *rplV*-R: 5′-GTGACCGCGAGAC GGAACCC-3′ and *rplD*-F: 5′-ATGGATCTCA AGCTCCTGAATG-3′, *rplD*-R: 5′- TCATCCCAG CATCTCCTCG-3′ respectively. For both cases, the final volume of the PCR mixture was 50 μL, distributed as follows: 25 μL of enzyme mix (*Taq* polymerase, 2.5 mM MgCl2, 15 mM Tris/HCl pH 8.3, 50 Mm KCl, 200 μM of each deoxynucleotide), 20 pmol of each primer (Macrogen, Seoul, Korea), and 5 μL of DNA extraction. Thus, the PCR conditions were: 95 °C for 5 min, followed by 55 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final elongation of 10 min at 72 °C. Amplified products (*rplV:* 408 bp; *rplD*: 619 bp) were gel recovered and sequenced as mentioned above.

3. Results

Both children presented fever, respiratory distress and flushed, while only one presented cough paroxysm (Table 1). One of the children was treated with amoxicillin plus cephalexin

Table 1
Clinical presentation of patients.

Symptoms	Patient 1	Patient 2
Paroxysm of coughing	–	+
Respiratory distress	+	+
Wheezing	–	–
ABOS	–	–
Lung collapse	–	–
Apnea	–	–
Pneumonia	+	–
Post-cough vomiting	+	–
Cyanosis	–	+
Flush	+	+
Fever	+	+
Convulsion	–	–
Feeding difficulties	+	+
Diarrhea	+	–
Vaccination status[a]	0	1

[a] Number of doses; ABOS: Acute bronchial obstructive syndrome.

Table 2
Antibiotic susceptibility (mm).

Antibiotic	Patient 1	Patient 2
Ampicillin	0	0
Amoxicillin	0	0
Cefotaxime	0	0
Erythromycin	0	0
Azithromycin	0	0
Clarithromycin	0	0
Chloramphenicol	14	16
Cotrimoxazole	14	30
Moxifloxacin	22	18
Tetracycline	16	16

(200 mg), while the other received ceftriaxone (100 mg). In both cases the treatment was successful and the children were discharged from hospital.

In both cases the PCR of the direct DNA extraction from nasopharyngeal swabs was positive for the presence of the *ptxA* gene and *IS418*, which showed a 100% of identity with that of *B. pertussis* (GenBank accession number NC_002929). Additionally, none of the other pathogens sought was detected, and thus, the patients were diagnosed with pertussis.

The antibiotic susceptibility analysis showed that the isolates present high levels of resistance to tested antibacterial agents, including all analyzed macrolides (Table 2).

Surprisingly, when the macrolide target encoding genes were amplified and sequenced, the results showed the presence of *A. xylosoxidans* A8 with 100% (*23S rRNA* gene) 99% (*rplD* gene) and 98% (*rplV* gene) of identity with the respective DNA sequences of *A. xylosoxidans* A8 (GenBank access CP002287.1), being even higher than the identity levels with the same DNA sequences of *B. pertussis* (Genbank access NC_002929) or other *Bordetella* spp. These results were also confirmed on PCR analysis of the grown microorganisms.

For further identification tool, we included the analysis of the *parC* gene sequence, which also showed that the closest related sequence was that of *A. xylosoxidans* A8, being 2–6 amino acid codons longer than other *Achromobacter* spp. and *Bordetella* spp. ParC sequences present in GenBank. Thus, despite the presence of 27 differences (96% of identity) in the encoding ParC DNA sequence both samples, only showed 3 different amino acid changes: S15P; D20G and Y66F.

Regarding the aforementioned macrolide targets no mutation was found in the *23S rRNA* gene and only 5 and 7 silent mutations were found in the *rplD* genes on comparison with the *A. xylosoxidans* A8 wild-type sequence, while 6 silent mutations plus the T134A transversion resulting in the amino acid change V45A was observed in both *rplV* genes.

4. Discussion

Achromobacter spp. are frequent environmental microorganisms, causing a series of opportunistic illnesses, including respiratory diseases [15,21] and possess a high evolutionary virulence potential, because of their phylogenetical proximity to *Bordetella*. In this line, the description of *A. xylosoxidans* in pediatric whooping cough patients is strongly suggestive of its implication as an etiological agent, especially as the isolate analysis showed the presence of the *ptxA* gene as well as IS418. A DNA fragment including the N-terminal of *parC* gene was also amplified to confirm these data. This gene was selected because the N-terminal section in *A. xylosoxidans* A8 has 5 to 6 more amino acid than the described ParC of *Bordetella* spp. Moreover, this sequence is also 2 to 5 amino acids longer than ParC of other *Achromobacter*.

The presence of non-*B. pertussis* microorganisms causing whooping cough has a direct implication in the lack or diminishing protection of vaccines and having a direct public health impact. Nonetheless, in this case only one child was partially vaccinated since the first vaccine dosage is provided at 2 months of life.

While IS418 is present with a variable copy number in different *Bordetella* genus members, the so-called pertussis toxin is exclusively produced by *B. pertussis* [22]. However, different members of the genus *Bordetella*, such as *Bordetella bronchiseptica* and *Bordetella parapertussis*, which have been involved as etiological causes of this illness, possess closely related non-functional genes [22]. Other *Bordetella* genus members, such as *B. holmesii,* which account for 0.4%–29% of all diagnosed pertussis cases [23,24], do not present the either pertussis toxin gene or close-related genes, a characteristic which has been used in different studies to facilitate the differentiation between *B. pertussis* and *B. holmesii* [25]. Thus, pertussis toxin functionality, although relevant, is not essential for the presence of pertussis-like symptoms. In our case no data about gene functionality was obtained, remaining to be established (studies ongoing).

The antimicrobial susceptibility analysis, showed high levels of resistance to macrolides. This is a matter of concern, since macrolides are considered as the treatment of choice for pertussis [26], and again highlight the need to correctly determine the etiologic agent, causing the illness. Along this line, it has been reported that different *Bordetella* species present different levels of macrolide resistance, which is especially high in *B. holmesii* [7].

Regarding macrolide targets, the only amino acid codon alteration with respect to *A. xylosoxidans* A8 wild-type sequences was observed in the *rplV* gene. Thus, the V45A amino acid codon substitution was observed in the protein L22 in both isolates. However, to the best of our knowledge, substitutions at this amino acid codon, or at equivalent positions in other microorganisms, have not been related to macrolide resistance. Nonetheless, the presence of this specific amino acid change may be a polymorphism without involvement in the development of macrolide resistance. Obviously, the presence of transferable mechanisms of macrolide resistance can not be ruled out. Thus, a GenBank search has showed the presence of the *ere*(A) gene in other members of the *Achromobacter* genus (GenBank access: DQ112222.1).

Interestingly, some silent DNA mutations were found in both strains in the *rplV*, *rplD* and *parC* genes. These close similarities between the two isolates, together with the short isolation frame time between the two isolates suggest the presence of a specific *A. xylosoxidans* A8 clone or a close related microorganism, with the ability to cause pertussis-like illness in the area. Taking this into account the recent report by Spilker *et al* [27] is of special interest. These authors reported the presence of several new putative *Bordetella*-like species, suggesting the presence of an undescribed genus closely related to both *A. xylosoxidans* and *Bordetella* spp.

In summary, the present results show the presence of *A. xylosoxidans* A8-like microorganism possessing the *ptxA* gene and *IS418*. Due the proximity of the *Achromobacter*

genome to that of *Bordetella* spp. causing whooping cough, in depth characterization of the isolates recovered is need. The present data highlight the possibility that closely related *B. pertusssis* microorganisms, not covered under the vaccine umbrella, might also result in cases of whooping cough, thereby requiring further surveillance to determine the extension and relevance of their pathogenic role in order to discriminate their real public health implication.

Conflict of interest statement

We declare that we have no conflicts of interest.

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